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# The role of the biomarker and the genetic polymorphism of endothelin-1 in pulmonary arterial hypertension among Egyptians

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## KEYWORDS

Polymorphism;  
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 hypertension

**Abstract** *Study objective:* This study analyses for the first time endothelin-1 (ET-1) level, and gene polymorphisms for endothelin-1 (EDN1 gene) in patients with pulmonary arterial hypertension (PAH) in Egypt.

*Design:* Cross-sectional study.

*Setting:* Large, tertiary care, Alexandria university teaching hospital, Chest Department.

*Subjects:* Thirty subjects with PAH with 30 control subjects.

*Methods:* Subjects were divided into two groups of matched age and sex were allocated. The first group consisted of thirty subjects,  $\geq 18$  years one group with no apparent evidence of disease free from pulmonary hypertension after full medical history, examination, and selected investigations (control group). The second group consisted of thirty subjects,  $\geq 18$  years, suffering from pulmonary hypertension. All subjects who had a documented pulmonary hypertension with routine Echo-doppler study were screened for endothelin-1(ET-1) and Gene polymorphism.

*Measurements and results:* This study analyzed the frequency and the potential role of endothelin-1 and gene polymorphisms, the +134 del/insA, located in the gene encoding for endothelin-1 (EDN1) in PAH. Thirty patients with pulmonary hypertension (12 [40%] men) were included in the study (Table 1). The mean age of the patients was  $53.5 \pm 12.8$  years range from 34 to 72 years. The two groups of patients and control subjects were matched as regard the age and gender. The endothelin-1 mean was  $1.8 \pm 1.3$  fmol/ml with range from 0.3 to 3.8 fmol/ml in the patients group. The endothelin-1 mean was  $0.7 \pm 0.05$  fmol/ml with range from 0.6 to 0.75 fmol/ml in the patients

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group. There was a significantly higher level of endothelin-1 in the group of pulmonary hypertension ( $p < 0.001$ ). For the groups of polymorphisms studied, there was three genotypes (GT, TT, and GG), no substantial differences in genotype and allele distributions for +134 del/insA located in EDN1 gene, between PAH patients and control population, were observed (DF = 1; C.I. = 95.0; and  $p = 0.226$ ). The genotype GG show the highest level of endothelin while the TT type show the lowest value of endothelin-1. Also, we found a significant relation between the higher endothelin-1 level and the lower oxygen saturation ( $p = 0.049$ ), and the higher meanPAP ( $p = 0.004$ ).

**Conclusions:** In conclusion, our findings suggest a potential link between endothelin-1 level and specific genotypes in the EDN1 gene and susceptibility for PAH with a worse haemodynamic profile. Further investigations are warranted to understand the molecular basis and to confirm the potential clinical importance of these findings on larger cohorts of patients with PAH. This will impact on the management of PAH of Egyptian patients in the near future.

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## Introduction

The term pulmonary arterial hypertension (PAH) describes a rare group of diseases characterized by raised pulmonary vascular resistance, resulting from vascular remodeling in the pre-capillary resistance arterioles. Left untreated, patients die from right heart failure, with a mortality approaching most serious cancers. Endothelin-1 (ET-1) is not only a potent vasoconstrictor, but causes proliferation of many of the vascular cells involved in vascular remodeling. Although produced mainly by the vascular endothelium, other cells such as smooth muscle, fibroblasts and macrophages are known sources of ET-1 when these cells are challenged by relevant stimuli. Plasma ET-1 levels are raised in patients with PAH and correlate with important clinical outcomes. Furthermore, ET-1 receptor antagonism has been demonstrated to improve both morbidity and mortality in conditions associated with PAH. Many articles in the literature supporting the role for ET-1 in the pathogenesis of PAH [1].

Pulmonary arterial hypertension (PAH) is a serious disease. Its prognostic is based on the functional status quantified by the NYHA class and the 6-min walking test, and the hemodynamic data. The algorithms of treatment are solely based on the hemodynamic data and the functional status [2].

ET-1 has an important role as vasoconstrictor and mitogen factor and its plasma levels have been found increased in PAH [3]. This molecule's effects are mediated by two receptors, ET<sub>A</sub> and ET<sub>B</sub>, found, respectively, on vascular smooth muscle and endothelial cells. They are G-protein-coupled receptors, and ET-1 vasoconstrictor effects depend on smooth muscle cell intracellular pH alkalisation and Ca<sup>2+</sup> increase [4]. The two receptors have different roles: in short terms, ET<sub>A</sub> is implicated only in vasoconstriction by increasing intracellular calcium, while ET<sub>B</sub> stimulation causes first a vasodilatation, followed by vasoconstriction and has also a role in ET-1 clearance. Polymorphic changes in EDN1 (encoding for endothelin-1, ET-1) and EDNRA (encoding for endothelin receptor type A; ET-A) genes have been suggested to play a role in human heart diseases, such as hypertension and dilated cardiomyopathy. To date, polymorphic changes in these genes have not been studied in patients with pulmonary artery hypertension (PAH) [5–7].

## Patients and methods

### Subjects

All subjects or their first degree relatives did sign an informed consent for the research. Thirty patients with diagnosis of pulmonary hypertension were included in the study. Thirty control subjects were included in the study. All subjects were subjected to the following: full history taking including past history of medical diseases and drug history. Also history of diabetes mellitus (confirmed by fasting blood glucose measurement), heart failure (confirmed by echocardiography), ischemic heart diseases and arrhythmias, obstructive lung disease, interstitial lung diseases, renal, and hepatic disease were included.

Subjects were divided into two groups of matched age and sex were allocated. The first group consisted of thirty subjects,  $\geq 18$  years one group with no apparent evidence of disease free from pulmonary hypertension after full medical history, examination, and selected investigations (control group). The second group consisted of thirty subjects,  $\geq 18$  years, suffering from pulmonary hypertension. All subjects who had a documented pulmonary hypertension with routine Echodoppler study were screened for endothelin-1 (ET-1) and gene polymorphism.

### Definition

We defined pulmonary hypertension (PAH) by measuring mean Pulmonary Artery Pressure (mPAP) and pulmonary hypertension (PAH) was defined as an increase in the mean pulmonary arterial pressure (mPAP) to  $> 25$  mmHg at rest [8].

### Measurement of serum big endothelin-1

Measurement of serum big endothelin-1 (Big ET). Blood samples were obtained early in the morning. The big endothelin-1 levels in serum was measured by commercially available enzyme linked immunosorbent assay (ELISA) kits (Biomedica Group) [9].

### Genotyping

Genomic DNA was extracted from peripheral whole blood samples collected in tubes with EDTA. The primers that were used in PCR amplification reaction were designed with Primer3 program, v.0.4.0, using genomic sequences from gene database on National Center for Biotechnology Information website, <http://www.ncbi.nlm.nih.gov>, (EDN1 Gene ID:

1955). We produced one amplicon for each polymorphism studied, by real time polymerase chain reaction (PCR): each reaction has a total volume of 25 µl, and was performed using 12 ng of genomic DNA, 0.5 mM dNTPs mix, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2 mM MgCl<sub>2</sub>, 2.5 pmol of each primer, 0.75 U of SYBR Green qPCR Master Mix® (Stratagene Max 3000). Primer sequences and annealing temperature for +134 del/insA (EDN1) were forward 5'-CTCCTGCAGTCC-CAGCTC-3', reverse 5'-CATGAGCAAATAATCCATTC-TG-3' (55 °C) corresponding to a 245-bp length fragment.

#### Genotype/phenotype analysis and statistical methods

Descriptive statistics including frequency, distribution, mean, and standard deviation were used to describe different characteristics. Hardy-Weinberg equilibrium was calculated by chi-square test with one degree of freedom. Genotype frequencies between groups were compared by the Mann-Whitney *U* test or the chi square-testing, as appropriate (2 groups), or the ANOVA test (3 groups). Univariate correlation was found by Pearson's analysis. A stepwise forward logistic multivariate model was used to test the effect of significant variables obtained by univariate models. Variables' rejection was employed at the 0.05 significance level. Data were analyzed using Statistical Package for the Social Sciences (SPSS) Version 18.0 for Windows (SPSS Inc, Chicago, IL, USA).

## Results

Thirty patients with pulmonary hypertension 12 men (40%) and 18 female (60%) were included in the study (Table 1). The mean age of the patients was  $53.5 \pm 12.8$  years range from 34 to 72 years. The two groups of patients and control subjects were matched as regard the age and gender (Table 1). There were 7 (23.3%) smokers. Table 2 demonstrates the clinical data of the studied subjects which shows that COPD was the highest cause for secondary pulmonary hypertension 10 (33.3%). Most of our patients had secondary pulmonary hypertension (80%). The mean oxygen saturation was  $88.76 \pm 3.68\%$ , mean pulmonary artery pressure was  $55.57 \pm 14.29$  mmHg, Ejection Fraction (EF) was  $56.03 \pm 9.81\%$ , and Fraction Shortening was  $30.0 \pm 5.60\%$ .

The endothelin-1 mean was  $1.8 \pm 1.3$  fmol/ml with range from 0.3 to 3.8 fmol/ml in the patients group. The endothelin-1 mean was  $0.7 \pm 0.05$  fmol/ml with range from 0.6 to 0.75 fmol/ml in the patients group (Table 3). There was a significantly higher level of endothelin-1 in the group of pulmonary hypertension ( $p < 0.001$ ). For the groups of polymorphisms studied, there was three genotypes (GT, TT, and GG), no substantial differences in genotype and allele distribu-

**Table 1** Pulmonary hypertension and control subjects demographic data.

Characteristic	PHT	Control	<i>p</i> value
Age (Years)	$53.56 \pm 12.8$	$51.6 \pm 13.9$	0.61
Gender			
M	12 (40%)	9 (30%)	0.33
F	18 (60%)	21 (70%)	
Smoking	7 (23.3%)	6 (20%)	0.68

M, male; F, female; PHT, pulmonary hypertension.

**Table 2** Clinical data of the studied patients.

	No.	%
DM	5	16.7
IHD	6	20.0
CHF	4	13.3
Liver disease	7	23.3
COPD	10	33.3
IPF	6	20.0
Bronchiectasis	3	10.0
Asthma	2	6.7
Steroid	6	20.0
Azathioprin	6	20.0
O <sub>2</sub> saturation		
Range	84.0–94.0	
Mean $\pm$ S.D.	$88.76 \pm 3.68$	
Type of hypertension		
Primary	6	20.0%
Secondary	24	80.0%
Mean PAP		
Range	38.0–77.0	
Mean $\pm$ S.D.	$55.57 \pm 14.29$	
EF%		
Range	38.0–76.0	
Mean $\pm$ S.D.	$56.03 \pm 9.81$	
FS%		
Range	18.0–44.0	
Mean $\pm$ S.D.	$30.0 \pm 5.60$	

**Table 3** Comparison between the two studied groups regarding Level of endothelin-1.

	Patients	Control
Range	.30–3.80	.60–.75
Mean	1.8250	.7050
S.D.	1.39121	.05309
T	12.03	
<i>p</i>	0.0001*	

\* Significant  $p < 0.05$ .

tions for +134 del/insA located in EDN1 gene, between PAH patients and control population, were observed (DF = 1; C.I. = 95.0; and  $p = 0.226$ ) (Table 4). The genotype GG shows the highest level of endothelin-1 while the TT type shows the lowest value of endothelin-1 and it was statistically significant ( $p = 0.0001$ ) (Table 5).

We studied the correlation between the different studied parameters (Table 6) and we found a significant relation between the higher endothelin-1 level and the lower oxygen saturation ( $p = 0.049$ ), and the higher meanPAP ( $p = 0.004$ ). Also, there was a significant correlation between lower oxygen saturation and the higher meanPAP ( $p = 0.011$ ). Additionally, there was a logic significant correlation between the EF% and FS% ( $p = 0.0001$ ).

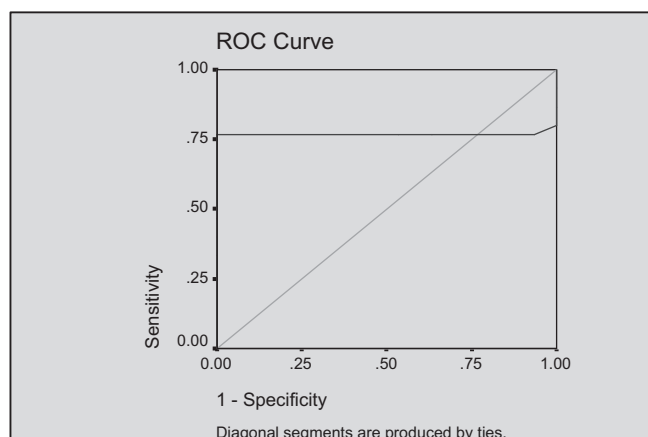
The ROC curve was done to determine the best sensitivity, specificity of endothelin-1 serum level as a diagnostic tool in PH. The sensitivity of endothelin-1 was 80% and specificity was 100% in detecting PAH at a cut off value of 0.525 fmol/ml.

**Table 4** Hardy–Weinberg analysis of the genotype in the two studied groups: comparison of genotypes and allele frequencies between controls and patients.

Gene polymorphism	Group		Total
	Patients group	Control group	
<i>GT</i>			
No.	9	15	24
%	30.0%	50.0%	40.0%
<i>TT</i>			
No.	3	4	7
%	10.0%	13.3%	11.7%
<i>GG</i>			
No.	18	11	29
%	60.0%	36.7%	48.3%
<i>Total</i>			
No.	30	30	60
%	100.0%	100.0%	100.0%
Hardy–Weinberg	Yes	Yes	
DF	1		
C.I.	95.0		
P	0.226		

DF = degree of freedom, CI = confidence interval.  $p$  = significance  $p < 0.05$ .

#### Coordinates of the curve



Test result variable(s): Enothelin

Positive if greater than or equal to(a)	Sensitivity	1 – Specificity
0.525	0.800	1.000

a = The smallest cutoff value is the minimum observed test value minus 1, and the largest cutoff value is the maximum observed test value plus 1.

#### Discussion

This study analyzed for the first times both serum endothelin-1 level (ET-1), and gene polymorphisms for endothelin-1 (EDN1 gene) among a sample of Egyptian patients with pulmonary arterial hypertension (PAH). The main finding of the study

was the association between the endothelin-1 level and the presence of the pulmonary hypertension, as well as, the association of GG genotype with higher endothelin-1 level in PHT patients. There was a significantly higher level of endothelin-1 in the group of pulmonary hypertension ( $p < 0.001$ ). For the groups of polymorphisms studied, there was three genotypes (GT, TT, and GG), no substantial differences in genotype and allele distributions for +134 del/insA located in EDN1 gene, between PAH patients and control population with a degree of freedom = 1; C.I. = 95.0; and  $p = 0.226$ . The genotype GG showed the highest level of endothelin-1 while the TT type showed the lowest value of endothelin-1. Also there was a significant relation between the higher endothelin-1 level and the lower oxygen saturation ( $p = 0.049$ ), and the higher mean PAP ( $p = 0.004$ ). As regards the sensitivity, specificity of endothelin-1; it was found that the sensitivity was 80% and specificity was 100% in detecting PAH at a cut off value of 0.525 fmol/ml.

The pathogenesis of PAH is dominated by a significant increase of pulmonary vascular resistance, due to cellular and biochemical modifications that lead to endothelial dysfunction, vasoconstriction, arterial wall thickening and thrombosis [10,11]. ET-1 has an important role as a vasoconstrictor and mitogen factor, and its plasma levels have been found increased in PAH [12]. ET-1 effects are mediated by two receptors, ET<sub>A</sub> and ET<sub>B</sub>, found, respectively, on vascular smooth muscle and endothelial cells [4]. In patients with moderate/severe PAH, pre-pro ET-1 mRNA levels in endothelial tissue of small arteries are significantly increased [13]. We found a significant relation between ET-1 level and severity of PAH. Previous studies on rats with PAH evidence endothelin receptor down regulation and a subsequent increase of vascular production of ET-1 and its mRNA, as well as a reduction of signal transduction [14]. Clinical investigations report an association between plasma ET-1 levels and PAH severity and outcome. All these data may suggest a key role for ET-1 and its receptors in PAH pathophysiology [15].

ET-1 and its receptors encoding genes polymorphisms have been previously studied [10]. That study looked at two polymorphisms: the +134 ins/delA situated in 5'UTR of EDN1 gene and the His323His located in the sixth exon of EDNRA gene [16]. The +134 ins/delA polymorphism correlates to endothelin-1 levels in a study ex vivo performed by Popowski et al. [17]. They found that the adenine insertion is related to an increasing level of endothelin expression (measured as pre-pro-ET-1 mRNA) with an evident increase in I/I homozygous carriers (3.9-fold respect to the wild-type D/D carriers). However, our study fails to demonstrate a role of this polymorphism in patients with PAH.

However, we found that genotype GG shows the highest level of endothelin-1 while the TT type shows the lowest value of endothelin-1. That could be due to racial factor of our population which is for the first time studied for PAH and possible role of gene polymorphism and also for the small sample size and that most of (80%) our patients have a secondary PAH which is different from other study where TT genotype was the most common type for PAH as their patients had primary PAH [18]. This also, explain the lower number of TT genotype as we have only 20% of our patients with primary PAH.

**Table 5** Relation between level of endothelin-1 and the genotype in the two studied groups.

Group	Gene polymorphism	Endothelin-1				<i>p</i>
		Min.	Max	Mean	S.D.	
Patients group	GT	.30	.85	.6333	.26101	19.415 .0001*
	TT	.40	.60	.4833	.10408	
	GG	1.10	3.80	2.6444	1.21980	
Control group	GT	.60	.75	.7033	.05499	1.02 0.365
	TT	.70	.75	.7250	.02887	
	GG	.60	.75	.7000	.05916	

\* Significant  $p < 0.05$ .**Table 6** Correlation between different studied parameters.

		Enothelin	Age	O <sub>2</sub>	Mean PAP	EF%
Age	<i>r</i>	.048				
	<i>p</i>	.802				
O <sub>2</sub>	<i>r</i>	-.362*	.058			
	<i>p</i>	.049	.759			
Mean PAP	<i>r</i>	.505**	.044	-.456*		
	<i>p</i>	.004	.816	.011		
EF%	<i>r</i>	.065	-.109	-.152	.087	
	<i>p</i>	.734	.567	.421	.646	
FS%	<i>r</i>	.054	-.029	-.122	-.013	.847**
	<i>p</i>	.777	.880	.521	.948	.0001

\* Correlation is significant at the 0.05 level (2-tailed).

\*\* Correlation is significant at the 0.01 level (2-tailed).

## Conclusion

Our findings suggest a potential link between endothelin-1 level and specific genotypes in the EDN1 gene and susceptibility for PAH with a worse haemodynamic profile. Further investigations are warranted to understand the molecular basis and to confirm the potential clinical importance of these findings on larger cohorts of patients with PAH. This will impact on the management of PAH of Egyptian patients in the near future. Understanding the role of EDN-1 in PAH has therapeutic significance since EDN1 receptor antagonists are currently few of the best possible options available in treatment of PAH. Studies have shown that addition of sildenafil to bosentan treatment could elicit additional hemodynamic benefits in PAH patients.

## Study limitations

Although our study and previous experimental and clinical studies [15,13] suggest a potential role of endothelin-1 level and EDN1 gene polymorphisms in the pathophysiology of PAH, the main limitation of the present study is the lack of demonstration of an in vitro functional role for this genotype. Pathogenesis of PAH is incompletely characterized, but it is likely to be multifactorial, involving the interaction between the genetic milieu and the environment of the individual. Endothelin-1 and endothelin receptor gene polymorphisms are probably disease modifiers, and their interactions with other genes and environmental features should be analyzed in future investigations. Moreover, the relatively small limited

cohort of patients and the inclusion of various PAH phenotypes may represent another potential bias.

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